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DOCUMENT-IDENTIFIER: US 6359054 B1
TITLE: Polynucleotide compositions for intramuscular administration

Brief Summary Paragraph Right (47):
A number of pluronics are designed to meet the following formula: ##STR14##

Brief Summary Paragraph Right (66):
Surfactant-Containing Polynucleotide Compositions. The invention also includes compositions of polynucleotides, cationic copolymer, and a suitable surfactant. The surfactant, should be (i) cationic (including those used in various transfection cocktails), (ii) nonionic (e.g., Pluronic or Tetronic), or (iii) zwitterionic (including betains and phospholipids). These surfactants increase solubility of the complex and increase biological activity of the compositions.

Brief Summary Paragraph Right (68):
Suitable non-ionic surfactants include n-Alkylphenyl polyoxyethylene ether, n-alkyl polyoxyethylene ethers (e.g., Tritons.TM.), sorbitan esters (e.g., Spans.TM.), polyglycol ether surfactants (Tergitol.TM.), polyoxyethylenesorbitan (e.g., Tweens.TM.), polysorbates, polyoxyethylated glycol monoethers (e.g., Brij.TM., polyoxylethylene 9 lauryl ether, polyoxylethylene 10 ether, polyoxylethylene 10 tridecyl ether), lubrol, copolymers of ethylene oxide and propylene oxide (e.g., Pluronic.TM., Pluronic R.TM., Teronic.TM., Pluradot.TM.), alkyl aryl polyether alcohol (Tyloxapol.TM.), perfluoroalkyl polyoxylated amides, N,N-bis[3-D-gluconamidopropyl]cholamide, decanoyl-N-methylglucamide, n-decyl .alpha.-D-glucopyranozide, n-decyl .beta.-D-glucopyranozide, n-decyl .beta.-D-maltopyranozide, n-dodecyl .beta.-D-glucopyranozide, n-undecyl .beta.-D-glucopyranozide, n-heptyl .beta.-D-glucopyranozide, n-heptyl .beta.-D-thioglucopyranozide, n-hexyl .beta.-D-glucopyranozide, n-nonanoyl .beta.-D-glucopyranozide 1-monooleyl-rac-glycerol, nonanoyl-N-methylglucamide, n-dodecyl .alpha.-D-maltoside, n-dodecyl .beta.-D-maltoside, N,N-bis[3-gluconamidepropyl]deoxycholamide, diethylene glycol monopentyl ether, digitonin, heptanoyl-N-methylglucamide, heptanoyl-N-methylglucamide, octanoyl-N-methylglucamide, n-octyl .beta.-D-glucopyranozide, n-octyl .alpha.-D-glucopyranozide, n-octyl .beta.-D-thiogalactopyranozide, n-octyl .beta.-D-thioglucopyranozide.

Brief Summary Paragraph Type 1 (21):
in which x, y, z, i, and j have values from about 2 to about 800, preferably from about 5 to about 200, more preferably from about 5 to about 80, and wherein for each R.sup.1, R.sup.2 pair, one is hydrogen and the other is a methyl group. Formulas (XXIV) through (XXVI) are oversimplified in that, in practice, the orientation of the isopropylene radicals within the B block will be random. This random orientation is indicated in formulas (XXVII) and (XXVIII), which are more complete. Such poly(oxyethylene)-poly(oxypropylene) block copolymers have been described by Santon, Am. Perfumer Cosmet., 72(4):54-58 (1958); Schmolka, Loc. cit. 82(7):25-30 (1967); Non-ionic Surfactants, Schick, ed. (Dekker, N.Y., 1967), pp. 300-371. A number of such compounds are commercially available under such generic trade names as "lipoloxamers", "poloxamers", "Pluronic.RTM.", and "synperonics." poly(oxyethylene)-poly(oxypropylene) polymers within the B-A-B formula are often referred to as "reversed" Pluronic.RTM., "Pluronic-R.RTM." or "meroxapol."

Brief Summary Paragraph Table (2):

Average Average # of # of oxypropylene oxyethylene CMC, Copolymer MW units, n.sup.1 units, n.sup.1 HLB .mu.M.sup.c L31 1100 17.1 2.5 5 1180 L35 1900 16.4 21.6 19 5260 L43 1850 22.3 12.6 12 2160 L44 2200 22.8 20.0 16 3590 L61 2000 31.0 4.5 3 110 L62 2500 34.5 11.4 7 400 L64 2900 30.0 26.4 15 480 F68 8400 29.0 152.7 29 480 L81 2750 42.7 6.2 2 23 P84 4200 43.4 38.2 14 71 P85 4600 39.7 52.3 16 65 F87 7700 39.8 122.5 24 91 F88 11400 39.3 207.8 28 250 L92 3650 50.3 16.6 6 88 F98 13000 44.8 236.4 28 77 L101 3800 58.9 8.6 1 2.1 P103 4950 59.7 33.8 9 6.1 P104 5900 61.0 53.6 13 3.4 P105 6500 56.0 73.9 15 6.2 F108 14600 50.3 265.4 27 22 L121 4400 68.2 10.0 1 1 P123 5750 69.4 39.2 8 4.4 F127 12600 65.2 200.4 22 2.8 .sup.1 The average numbers of oxyethylene and oxypropylene units were calculated using the average molecular weighs. The hydrophilic-lipophilic balance (HLB) of the copolymers were determined by the manufacturer (BASF Co.). The critical micellization concentrations (CMC) were determined by the surface tension method described in Kabanov et. al., Macromolecules 28: 2303-2314 (1995).

Detailed Description Paragraph Right (1):
This experiment introduced plasmid p.beta.-Gal into NIH 3T3 cells, a mouse mammary tumor cell line. Plasmid p.beta.-Gal comprises plasmid pUC19 (available from the Institute of Gene Biology, Russian Academy of Sciences) into which a hybrid of a eukaryotic transcription unit and a E. coli .beta.-galactosidase has been incorporated. With this plasmid, the efficiency of cell uptake can be measured by measuring .beta.-galactosidase activity extractable from the treated cells. The copolymer utilized was a triblock copolymer of formula (XIV) wherein x plus z was 51 and y was 39 (hereinafter "Pluronic A"). The polycation used was poly(N-ethyl-4-vinylpyridinium bromide) ("pEVP-Br"). A 10 .mu.g/ml solution of p.beta.-Gal (predominantly supercoiled) was prepared in a solution of PBS containing 10 mg/ml of Pluronic A and 45 .mu.g/ml of pEVP-Br. These amounts were calculated to provide a ratio of polycation basic groups to plasmid phosphate groups of about 10. The ratio of Pluronic A to DNA was about 10.sup.4. This stock preparation was filter sterilized and a portion was diluted ten fold with serum-free Dulbecco's Modified Eagle's Medium ("DMEM"), so that the concentration of p.beta.-Gal was 1 .mu.g/ml. This solution was the "Pluronic A transfecting medium."

Detailed Description Paragraph Right (3):
Aliquots of washed cells that were to be transformed by the method of the invention were suspended at a concentration of 10.sup.6 cells/ml in Pluronic A transfecting medium. The suspended cells were incubated for 2 hours at 37.degree. C. and under 5% CO.sub.2. The cells were then washed with fresh medium and re-plated.

Detailed Description Paragraph Right (6):
In these experiments, transfection efficiencies with MDCK cells (derived from canine kidney) were examined. As above, p.beta.-Gal was the indicator polynucleotide. The polycation component of the polynucleotide comprised a copolymer of N-ethyl-4-vinylpyridinium bromide and N-cetyl-4-vinylpyridinium bromide, the monomers incorporated in a molar ratio of 97:3, respectively (hereinafter "pEVP-co-pCVP-Br"). The block copolymer comprised a triblock copolymer of formula (XIV) wherein x+z was 18, and y was 23 (hereinafter "Pluronic B"). A Pluronic B transfecting solution of 1 .mu.g/ml p.beta.-Gal, 3 .mu.g/ml PEVPco-pCVP-Br, and 1% (w/v) Pluronic B was prepared in Example 1. The ratio of polycation basic groups to nucleotide Phosphates was about 7. The weight ratio of Pluronic B to p.beta.-Gal was about 5.times.10.sup.3.

Detailed Description Paragraph Right (7):
MDCK cells were plated at 8-10.sup.5 cells per plate onto 90 mm plates and incubated overnight under serum-containing growth medium. The serum containing medium was then replaced with serum-free medium, and the cells were incubated at 37.degree. C., under 5% CO.sup.2 for 24 hours. For the cells to be treated with polynucleotide complex, the medium was then replaced with 5 ml Pluronic B transfecting solution. The cells were incubated, with gentle rocking, at 37.degree. C., under 5% CO.sub.2 In control experiments, cells were transfected with polynucleotide complex, the medium was then replaced with 5 ml Pluronic B transfecting solution. The cells were incubated, with gentle rocking, at 37.degree. C., under 5% CO.sub.2, for 2 hours. In control experiments, cells were transfected using the calcium phosphate procedure as described above (except that plated cells, not suspended cells, were transfected).

Detailed Description Paragraph Right (8):

After treatment with Pluronic B transfecting solution or calcium phosphate, the cells were washed 5-6 times with fresh medium. They were then incubated in DMEM containing 10% FCS for 48 hours at 37.degree. C., under 5% CO.sub.2. After the first 16 hours of this incubation, the medium was replaced. After the incubation, the cells were washed with PBS, released from their plates by trypsinization, and again washed with PBS. .beta.-Galactosidase was measured as described for Example 1. The results were as follows:

Detailed Description Paragraph Right (9):
In these experiments, transfection efficiencies with Chinese hamster ovary (CHO) cells were examined. The polynucleotic component of the polynucleotic complex was p.beta.-Gal. The polycation component comprised pEVPBr. The block copolymer comprised an octablock copolymer formula (XVII), wherein i was equal to 10 and j was equal to 12 (hereinafter "Pluronic C" available from BASF). A Pluronic C transfecting solution of 1 .mu.g/ml p.beta.-Gal, 4 .mu.g/ml pEVP-Br, and 1% (w/v) Pluronic C was prepared as in Example 1. The ratio of basic groups to nucleotide phosphates was 10. The weight ratio of Pluronic C to p.beta.-Gal was 10.sup.3. The transfection protocol was the same as that used in Example 2. The results were as follows:

Detailed Description Paragraph Right (31):
The 3'-terminal of the purified Oligo A was oxidized with periodate to create an aldehyde and conjugated by reductive alkylation with a hexamethylene-diamine linker, creating an amine derivative. See Che-Chung et al., Biochem. Intemat., 25:767 (1991); Vinogradov et al., BBRC, 203:959 (1994). "Pluronic A", a block copolymer of formula (XIV) (x=25, y=38, z=25) was similarly oxidized to create terminal aldehydes. The amine derivative (1 mg) was dissolved in 100 .mu.l of 0.1 M borate buffer (pH 9.0) and mixed with 2 mg of the Pluronic A derivative. 1.5 mg of sodium cyanoborohydride was added to the mixture to reduce the Schiffs bases formed between the amine and aldehyde groups. This reaction was allowed to proceed for 12 hours at 4.degree. C. The polymeric product of this reaction was isolated by gel filtration chromatography on Sephadex LH-20, utilizing 90% aqueous isopropanol as the eluent. The conjugate so obtained is referred to hereinafter as "Oligo A Conjugate."

Detailed Description Paragraph Right (57):
Following the procedure of Example 28 but substituting 24 g of poly(ethylene glycol) by the same amount of Pluronic L61 (BASF Co.) and using a molar ratio of activated Pluronic L61 to free aminogroups of polyethyleneimine 0.3:1.0, there is obtained in 22% yield a grafted polyethyleneimine copolymer in which 8% of free aminogroups are substituted with Pluronic L61.

Detailed Description Paragraph Right (58):
Following the procedure of Example 28 but substituting 24 g of poly(ethylene glycol), by the same amount of Pluronic P85 and using a molar ratio of activated Pluronic P85 to free aminogroups of polyethyleneimine 0.3:1.0 there is obtained in 70% yield a grafted polyethyleneimine copolymer in which 11% of free aminogroups of polyethyleneimine are substituted with Pluronic P85.

Detailed Description Paragraph Right (59):
Following the procedure of Example 28 but substituting 24 g of poly(ethylene glycol), by the same amount of Pluronic P123 (BASF Co.) and using a molar ratio of activated Pluronic P123 to free aminogroups of polyethyleneimine 0.3:1.0 there is obtained in 30% yield a grafted polylysine copolymer in which 9% of free aminogroups are substituted with Pluronic P123.

Detailed Description Paragraph Right (60):
Following the procedure of Example 28 but substituting 24 g of poly(ethylene glycol), by the same amount of Pluronic F38 (BASF Co.) and using a molar ratio of activated Pluronic F38 to free aminogroups of polyethyleneimine 0.3:1.0 there is obtained in 40% yield a grafted polylysine copolymer in which 9% of free aminogroups are substituted with Pluronic F38.

Detailed Description Paragraph Right (61):
Following the procedure of Example 28 but substituting polyethyleneimine by polyethyleneimine modified with Pluronic L123 (BASF Co.) obtained in Example 35 and using a molar ratio of activated poly(ethylene glycol) to free aminogroups of modified

polyethyleneimine 0.4:1.0 there is obtained in 20% yield a grafted polyethyleneimine copolymer in which 9% of free aminogroups are substituted with Pluronic L123 and 30% of groups are substituted with poly(ethylene glycol).

Detailed Description Paragraph Right (73):
These experiments are performed in Cos-7 cells and carried out as follows; Cos-7 cells are used and are seeded at 7.times.10.sup.5 per well in 12-well plate (Costar) and allowed to rest 24 hours before transfection (confluenly at 70%). Three .mu.g of pGL3-Luc SV40 is formulated with the different polymers at various N/P ratios. The transfection mixture is prepared as follows; to an eppendorf tube containing 100 .mu.l of DMEM supplemented with 1% Hepes the following is added; 30 .mu.l of DNA at 0.1 mg/ml, 35 .mu.l of polymer to be tested at various concentrations to get a variety of N/P ratios. The transfection mixture is allowed to incubate 5 minutes before completing to 1 ml with complete DMEM (10% FBS, 1% Hepes, 1% penicillin-streptomycin). Five hundred .mu.l of the transfection mixture is added per well. Following a 4-hours transfection at 37.degree. C. and under a 5% CO.sub.2 atmosphere, the cells are rinsed with PBS and allowed to rest overnight in 1 ml of complete DMEM before being harvested to perform the luciferase assay according to Promega Corporation's recommendation. Briefly, the cells are lysed on ice for 30 minutes and then centrifuged at 13 000 g for 2 minutes. The supernatents are kept and analyzed for luciferase activity. The assay is performed as follows: 20 .mu.l of supernatent is added to luminometric tubes containing 100 .mu.l of luciferase substrate. Light emission is measured with a luminometer (Berthold) for a period of 5 seconds. The data is reported in relative light units per second and normalized for proteins with the BiCinchoninic Acid assay kit (Sigma). The results show that pluronic P123 conjugated to PEI improves transfection of CMV-Luc compared to PEI alone suggesting that the block copolymer moiety is advantageous for a better transfection. Note that P123 alone does not transfect cells and is totally inefficient like CMV-Luc alone. This observation is in contrast to the data shown in example 44 where P123 is used to improve gene expression in muscle.

Detailed Description Paragraph Right (74):
CMV-Luc (50 .mu.g) or oligonucleotides (100 .mu.g) are resuspended in a volume of 200 ul containing various block copolymers-based formulations and injected i.v. into C57B1/6 (6-8 week-old) female mice. Twenty-four hours following the injection the mice are sacrificed to harvest various organs in which luciferase activity is measured or in which oligonucleotide accumulation is determined. For plasmid DNA, all major organs are rapidly homogenized with a tissue grinder (Kontes Glass Co.) in cell lysis buffer (Promega Corporation) supplemented with protease inhibitors. The extraction mixture is kept on ice for 30 minutes and then centrifuged at a maximum speed for 2 minutes. The supernatents are kept and analyzed for luciferase activity. The assay is done as follows: 20 .mu.l of supernatent is added to luminometric tubes containing 100 .mu.l of luciferase substrate (Promega Corporation). Light emission is measured with a luminometer (Berthold) for a period of 5 seconds. The data is reported in pg of luciferase per mg of proteins. For oligonucleotides, the major organs are extracted twice with phenol:chloroform and precipitated with ethanol before quantification. The result show that with conventional liposomal formulation and PEI that gene expression is concentrated in the lungs which is a factor known to increase risks of pulmonary embolism. However, gene expression is redirected to liver when formulated with PEI conjugated to a hydrophobic block copolymer such as P123. In addition, when P123 is used alone, gene expression in various organs is very low except in muscle tissue. For oligonucleotides, the accumulation is observed is kidneys when a hydrophobic carrier (PEI conjugated to PEG) is used and is redirected to liver when a hydrophobic carrier (P85-PEI/P85) is used. Various and a multitude of mixture of block polymers can be prepared to give a wide range of hydrophobic and hydrophylic balances that can redirect gene expression and oligonucleotides accumulation in various regions of the body.

Detailed Description Paragraph Table (1):
Treatment Relative Enzyme Activity .+-. SEM (n = 4) Pluronic A 320 .+-. 42 Calcium Phosphate 17 .+-. 5 Precipitation

Detailed Description Paragraph Table (2):
Relative .beta.-galactosidase activity .+-. Treatment SEM (n = 4) Pluronic B 910 .+-. 45 Calcium Phosphate Precipitation 81 .+-. 17

Detailed Description Paragraph Table (3):
Relative .beta.-galactosidase Treatment activity .+-. SEM (n = 4) Pluronic B 910 .+-. 45 Calcium Phosphate Precipitation 81 .+-. 17

Detailed Description Paragraph Table (17):
Transfection mixture Luciferase signal (RLU/s/ug proteins) CMV-Luc alone 15 .+-. 4 CMV-Luc + P123-PEI/123 1789456 .+-. 45789 CMV-Luc + P123 26 .+-. 6 CMV-Luc + PEI 678543 .+-. 32591

Detailed Description Paragraph Table (18):
Organs with the highest luciferase signal or with the highest accumulation of Transfection mixture oligonucleotides CMV-Luc alone none CMV-Luc + P123-PEI/P123 Liver CMV-Luc + P123 Muscle CMV-Luc + PEI Lungs CMV-Luc + Liposome (Dotap-chol) Lungs Oligo alone Lungs and Liver Oligo + PEI conjugated to PEG Kidneys Oligo + P85-PEI/P85 Liver

Detailed Description Paragraph Table (20):
PLURONIC P123 P123 (%) RLU/s/T.A. muscle 0 31005 .+-. 5619 0.0007 6052 .+-. 1778 0.007 100499 .+-. 30455 0.07 => 130113 .+-. 46871 0.7 5368 .+-. 1505 7 160 .+-. 23

Detailed Description Paragraph Table (21):
PLURONIC P123 P123 (%) RLU/s/T.A. muscle 0 31005 .+-. 5619 0.0007 6052 .+-. 1778 0.007 100499 .+-. 30455 0.07 => 130113 .+-. 46871 0.7 5368 .+-. 1505 7 160 .+-. 23

CLAIMS:

11. A pharmaceutical composition according to claim 1 wherein said polynucleotide, viral vector, or polynucleotide derivative thereof is plasmid DNA.

23. The method according to claim 13 wherein the block copolymer comprises the block copolymer Pluronic L61.